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Inhibitory Effect of Scopoletin Isolated from *Sorbus commixta* on TNF-α-Induced Inflammation in Human Vascular Endothelial EA.hy926 Cells through NF-κB Signaling Pathway Suppression

Hye Ryung Kang[1], Hyo Jung Kim[1], Bomi Kim[1], Sun-Gun Kim[1], Jai-Hyun So[1], Soo Jeong Cho[2] and Hyun Sook Kwon[1]*

[1]*National Institute for Korean Medicine Development, Gyeongsan 38540, Korea*
[2]*Department of Pharmaceutical Engineering, Gyeongnam National University of Science and Technology, Jinju 52725, Korea*

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Sorbus commixta Hedl. has traditionally been used as a remedy for cough, asthma, and other bronchial disorders. In this study, three major triterpenoids－lupeol, β-sitosterol, and ursolic acid and a coumarin, scopoletin, were isolated from a $CHCl_3$-soluble fragment of the bark of *S. commixta*. Their structures were identified by spectroscopic analyses, including mass spectrometry (MS), 1D-, and 2D- nuclear magnetic resonance spectroscopy (NMR), as well as by comparing the data with data reported in the literature. Scopoletin was isolated from this plant for the first time. It is a nutraceutical compound contained in many plants that has been reported to exert diverse biological activities, including anti-inflammatory effects. This study examined the inhibitory effect of scopoletin on TNF-α-induced vascular endothelial inflammation. Unlike the marginal impact of other compounds against low-density lipoprotein (LDL) oxidation and vascular endothelial inflammation, scopoletin showed remarkable activity on LDL oxidation (IC_{50} = 10.2 μM) and exerted vascular anti-inflammatory effects in EA.hy926 human endothelial cells activated by TNF-α. It suppressed the expression of adhesion molecules, such as ICAM-1, VCAM-1, and E-selectin, and blocked the adhesion between THP-1 monocytes and EA.hy926 endothelial cells. It also inhibited TNF-α-induced NF-κB translocation from the cytosol to the nucleus. Moreover, IκBα phosphorylation, which was increased by TNF-α treatment, was reduced after treatment with scopoletin. Thus, scopoletin inhibited TNF-α-induced vascular inflammation in endothelial cells by suppressing the NF-κB signaling pathway. These results demonstrate that owing to its anti-inflammatory activity in the vascular endothelium, scopoletin has the potential to inhibit atherosclerosis development.

Key words : Adhesion molecules, inflammation, scopoletin, *Sorbus commixta*

Introduction

Atherosclerosis is a chronic vascular disease described as endothelial dysfunction, increase of cell adhesion molecules, and accumulation of foam calls, smooth muscle cells, and fibrous tissue in the intima area. The process of atherogenesis is not fully understood yet, but it is well known that inflammation plays a crucial role in all stages of atherosclerosis [10, 19, 20]. Previous studies reported that adhesion of circulating monocytes to the injured endothelial layer, invasion of monocytes into the vessel wall, and differentiation into macrophages are early events in the development of atherosclerosis [10, 19, 20]. The adhesion of monocytes onto endothelial cells can be controled by the expression of cell adhesion molecules, including intercellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin [10]. Furthermore, the expressions are upregulated by a proinflammatory cytokine such as tumor necrosis factor-α (TNF-α) [10]. Besides, nuclear factor-κB (NF-κB) is a central mediator in adhesion molecules expression and monocyte adhesion. In normal condition, NF-κB is localized in the cytoplasm and bind to its inhibitor protein, IκB. When it is activated by a variety of external stimuli, such as TNF-α, the IκB is phosphorylated and degraded in proteasome [2, 4, 7]. This action results in release of NF-κB, which then translocates to the nucleus and binds to its promoter κB binding site and transcribes a number of inflammatory genes [2, 4, 7].

Sorbus commixta Hedl. (Rosaceae) is well known as medic-

***Corresponding author**
Tel : +82-53-810-0389, Fax : +82-53-810-0301
E-mail : sook4269@naver.com

inal plant in Korea, China, and Japan. The diverse pharmacological effects of *S. commixta* on antioxidative [1], anti-inflammatory [24], anti-lipid peroxidative [14], anti-atherogenic [21, 22], and vasorelaxant activities [23] have been reported. Scopoletin (6-methoxy-7-hydroxycomarin), an active component of *S. commixta*, showed anti-inflammatory [13], anti-allergy [5] and anti-angiogenic properties [17]. The effect of scopoletin on the expressions of proinflammatory mediators has been estimated by several studies. Scopoletin reduced expression levels of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), interleukin-1β (IL-1β), interleukin-6 (IL-6), and TNF-α in Raw264.7 cells stimulated with lipopolysaccharide [13]. Although the prior reports focused on anti-inflammatory effects of scopoletin in several cell types, its anti-inflammatory effects in EA.hy926 human vascular endothelial cells have not been clarified. In the present study, we did isolate pure compound, scopoletin, from bark MeOH extracts of *S. commixta*, and evaluated whether scopoletin inhibits the expression of cellular adhesion molecules and monocyte adhesion onto EA.hy926 human vascular endothelial cells and nuclear NF-κB are targets for the inhibitory actions of scopoletin on adhesion molecule expression.

Materials and Methods

Plant material

The *S. commixta* Hedl. was collected on December 2017, in Gyeongsangnam-do Agricultural Research & Extension Service, Medicinal Source Research Institute, Hamyang district of Korea.

Instruments

Melting points were measured on a Thomas Scientific Capillary Melting Point Apparatus and are uncorrected. IR spectra were recorded on a Bruker IFS66 infrared Fourier transform spectrophotometer (KBr). NMR experiments were conducted on Bruker AM 300 and 500 (^{1}H-NMR at 300 and 500 MHz, ^{13}C-NMR at 75 and 125 MHz) spectrometer with tetramethylsilane (TMS) as the internal standard. EIMS were recorded on a Jeol JMS-700 instrument operated at 70 eV. TLC analysis was performed on Kieselgel 60 F_{254} (Merk, Darmstadt, Germany) plates. Silica gel (Merck, 70-230 and 230-240 mesh) and Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden) were used for column chromatography.

Extraction and isolation

The dried bark of *S. commixta* (500 g) was extracted with MeOH (5000 mL) at room temperature for three days. The MeOH extracts (20 g) was evaporated to dryness and suspended in H_2O, then it was partitioned with $CHCl_3$ (10 g) and BuOH (5.5 g). The $CHCl_3$-soluble fraction was chromatographed on a silica gel column eluted with a gradient of 100% $CHCl_3$ to 100% MeOH to afford ten fractions (F1 to F10). F1 (2 g) was chromatographed over silica gel using *n*-hexane:EtOAc (19:1→1:1) to give ten major subfractions (F1-1 to F1-10). F1-3 (900 mg) was subjected to column chromatography on Sephadex LH-20 to yield five fractions (F1-3-1 to F1-3-5). F1-3-3 was recrystallized from MeOH to give compound **1** (108.2 mg). F1-7 (0.95 g) was purified by repeated silica gel column chromatography using *n*-hexane:EtOAc gradient (19:1→1:1) to afford eight fractions (F1-9-1 to F1-9-8). Compound **2** (94.1 mg) was crystallized in $CHCl_3$ from F1-9-2. TLC analysis of F1-9-8 indicated the presence of only one major component. Compound **3** (105.6 mg) was purified by sephadex LH-20 column chromatography in 100% MeOH solvent condition. F5 (0.94 g) was chromatographed over silica gel using *n*-hexane:EtOAc gradient (4:1→1:1) to afford ten fractions (F5-1 to F5-10). Of these, F5-9 (0.8 g) was chromatographed over silica gel with $CHCl_3$:Me_2CO gradient (99:1→1:1) to produce six fractions (F5-9-1 to F5-9-6). Further chromatographic separation of F5-9-5 was carried out by preparative TLC to give compound **4** (50 mg).

Lupeol (1)

White amorphous powder; mp 210℃; EI/MS *m/z* 426 $[M]^+$; IR λ_{max} 3300, 1650 and 1500 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 0.76 (3H, s, H-24) 0.79 (3H, s, H-28), 0.83 (3H, s, H-25), 0.95 (3H, s, H-27), 0.96 (3H, s, H-23), 1.03 (3H, s, H-26), 1.68 (3H, s, H-30), 1.92 (1H, m, H-21), 2.40 (1H, m, H-19), 3.20 (1H, dd, *J*=5.4, 9.9 Hz, H-3), 4.57 (1H, s, H-29b), 4.69 (1H, s, H-29a); ^{13}C-NMR (75 MHz, $CDCl_3$) see Table 1.

β-sitosterol (2)

White amorphous powder; mp 140°C; EI/MS *m/z* 414 $[M]^+$; IR λ_{max} 3421, 2937, 1643, 1462 and 1380 cm^{-1}; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 0.70 (3H, s, H-18), 0.82 (3H, s, H-27), 0.84 (3H, s, H-26), 0.86 (3H, s, H-29), 0.95 (3H, s, H-21), 1.0 (3H, s, H-19), 3.54 (1H, m, H-3), 5.35 (1H, d, *J*=5.2 Hz, H-6); ^{13}C-NMR (125 MHz, $CDCl_3$) see Table 1.

Table 1. ^{13}C-NMR data for compound 1-3

Position	1[a]	2[b]	3[b]
1	38.7	37.2	38.6
2	27.4	31.6	27.3
3	78.8	71.8	77.1
4	38.8	42.3	36.9
5	55.3	140.7	55.1
6	18.3	121.7	18.3
7	34.3	31.9	33.0
8	40.8	31.9	38.8
9	50.4	50.1	47.1
10	37.1	36.5	36.8
11	20.9	21.1	23.2
12	25.1	39.8	124.9
13	38.0	42.3	138.5
14	42.8	56.7	42.0
15	27.4	24.3	27.9
16	35.6	28.2	24.1
17	42.9	56.0	47.3
18	48.3	11.8	52.7
19	47.9	19.4	38.8
20	150.7	36.1	38.7
21	29.8	18.79	30.5
22	40.0	33.9	36.6
23	28.0	26.1	28.6
24	15.4	45.8	15.5
25	16.1	29.1	16.4
26	16.0	19.8	17.3
27	14.5	19.0	23.6
28	18.0	23.0	178.6
29	109.3	11.9	17.2
30	19.3	-	21.4

[a]75MHz in $CDCl_3$ at 25℃. [b]125MHz in $CDCl_3$ at 25℃.

Ursolic acid (3)

White amorphous powder; mp 279℃; EI/MS *m/z* 456 $[M]^+$; IR λ_{max} 3420, 2920, 1690, 1450 and 1376 cm^{-1}; ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 0.68 (3H, s, H-25), 0.76 (3H, s, H-29), 0.82 (3H, d, *J*=6.3 Hz, H-30), 0.87 (3H, s, H-24), 0.92 (6H, s, H-26, 27), 1.05 (3H, s, H-23), 3.02 (1H, dd, *J*=4.9, 10.0 Hz, H-3), 5.13 (1H, s, H-12); ^{13}C-NMR (125 MHz, DMSO-d_6) see Table 1.

Scopoletin (4)

Yellow amorphous powder; mp 204℃; EI/MS *m/z* 192 $[M]^+$; IR λ_{max} 3339, 1704 and 1566 cm^{-1}; ^{1}H-NMR (500 MHz, CD_3OD) δ: 3.88 (3H, s, OCH_3), 6.14 (1H, d, *J*=9.3 Hz, H-3), 6.70 (1H, s, H-8), 7.03 (1H, s, H-5), 7.82 (1H, d, *J*=9.3 Hz, H-4); ^{13}C-NMR (125 MHz, CD_3OD) δ: 57.0 (C-6, OCH_3), 104.5 (C-8), 109.9 (C-5), 111.6 (C-3), 112.0 (C-4a), 146.6 (C-4), 148.3 (C-6), 152.4 (C-8a), 155.9 (C-7), 164.8 (C-2).

All compounds (**1-4**) were dissolved in DMSO at the concentration 100 mM as the stock solution, which were stored at -20℃ until their anti-vascular inflammatory analysis.

LDL oxidation assay

Assay of LDL oxidation was measured at the microplate reader from the absorbance of thiobarbituric acid reactive substances (TBARS) products. Briefly, 0.1 ml human LDL (Merck, Darmstadt, Germany) of 0.1 mg/ml (diluted in 10 mM PBS) was mixed with samples (0-0.1 mM), followed by addition of copper sulfate ($CuSO_4$, final concentration 0.05 mM) as a LDL oxidation generator. After incubation at 37℃ for 6 hr, mixture was added with 50 μl of 20% tricarboxylic acid (TCA) and 0.67% thiobarbituric acid (TBA, diluted in 0.05 M NaOH), and then heated at 37℃ for 40 min, cooled. The absorbance of mixture was measured at 532 nm by microplate reader (Sunrise; Tecan, Grödig, Austria).

Cell culture and treatments

Human vascular endothelial cells (EA.hy926) and human THP-1 monocytes were obtained from American Type of Culture Collection (Manassas, USA). The cells maintained in Dulbecco's modified eagle medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) at 37℃ in 5% CO_2/95% air and passaged every other day. For cell-based experiments, EA.hy926 cells (1.5×10^4 cells/well) were treated with control (con., non-treated), TNF-α (30 ng/ml) and TNF-α plus samples (0-40 μM). Cells were preincubated with samples for 2 hr before addition of TNF-α (Enzo, NY, USA) for the indicated times.

Western blotting

For whole cell lysates preparation, EA.hy926 cells were washed with cold PBS and lysed in RIPA lysis buffer (Wako, Osaka, Japan) and for nuclear and cytosolic extractions, cells were lysed using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Waltham, MA, USA) according to manufacturer's protocol. Protein concentration was determined using Pierce™ BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). Lysates were separated by electrophoresis on 10% SDS-polyacrylamide gel and transferred onto PVDF membrane. Membranes were incubated with anti-ICAM-1, anti-NF-κB, anti-phospho-IκBα, anti-β-actin or anti-Lamin B antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight at 4℃, and then incubated horse radish peroxidase-linked secondary anti-

bodies (Santa Cruz, Dallas, TX, USA) for 2 hr. Target protein bands were detected using a SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Scientific, Waltham, MA, USA) and chemiluminescence image analyzer (Fuji Film LAS-400, Tokyo, Japan). Densitometry of the bands was analyzed by Image Studio Software (LI-COR, Inc., Lincoln, NE, USA).

Real-time polymerase chain reaction (PCR)

Total RNA was extracted from cultured cells with the Qiazol lysis reagent (Qiagen, Hilden, Germany) and RNA extracts (0.2 μg) were reverse-transcribed into cDNA using Revert Aid™ First cDNA Kit (Thermo Scientific, Waltham, MA, USA) as described in the manufacturer's directions. The reaction was amplified with StepOnePlus™ real-time PCR system (Applied Biosystems, CA, USA) for 40 cycles with denaturing at 95℃ for 15 sec, annealing at 60℃ for 60 sec and elongation at 95℃ for 60 sec. Primer sequences were as follows: ICAM-1 (forward, 5′-TATGGCAACGACTCCTT CT-3′; reverse, 5′- CATTCAGCGTCACCTTGG-3′), VCAM-1 (forward, 5′-AGTTGAAGGATGCGG GAGTA-3′; reverse, 5′-AGAGCACGAGAAGCTCAGGA-3′), E-selectin (forward, 5′-GAGGCCAGTGCTTATTGTCA-3′; reverse, 5′-CATTCAGCG TCACCTTGG-3′) and GAPDH (forward, 5′-CAACGGATTT GGTCGTATTG-3′; reverse, 5′-GATGACAAGC TTCCCGTT CT-3′). The levels of gene expression were normalized to GAPDH as an internal control and quantified using the comparative threshold cycle (Ct) method.

Adhesion assay

THP-1 cells were stimulated with TNF-α for 18 hr and then were labeled with 10 μM BCECF-AM (Invitrogen, Paisley, UK) for 1 hr. The labeled THP-1 cells (7×10^4 cells/well) were then added to the EA.hy926 cells which were treated with TNF-α and/or samples, followed by incubated for 45 min at 37℃. After incubating, non-adherent cells were removed by washing with PBS and quantification of adherent cells was detected by fluorescence intensity at 485 and 535 nm of excitation and emission in a fluorescence microplate reader (Victor X5; PerkinElmer, Waltham, MA, USA).

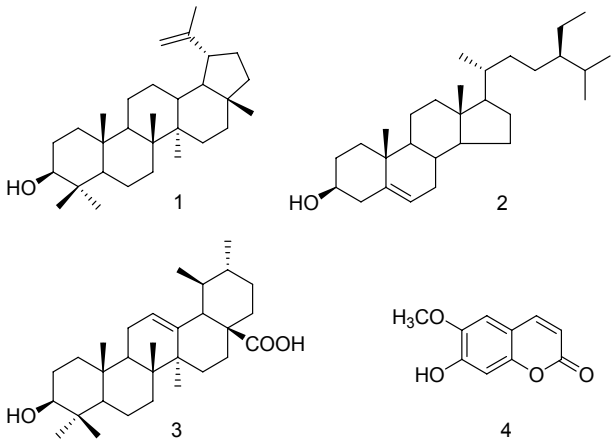

Fig. 1. Structures of compounds 1-4.

Statistical analysis

All data values are expressed as mean ± standard deviation (SD). Significant differences between groups were analyzed using analysis of variance (ANOVA) by PASW statistics (SPSS Inc., Chicago, IL, USA) followed by Duncan's multiple range test to determine statistical difference among groups. Statistical significance was defined as $p<0.05$.

Results and Discussion

Isolation and structural elucidation of compounds

Three triterpenoids, lupeol (**1**), β-sitosterol (**2**), ursolic acid (**3**), and a coumarin, scopoletin (**4**) were isolated from a $CHCl_3$-soluble fraction of *S. commixta* bark by repeated silica gel column chromatography.

Compound **1** was obtained as white amorphous powder and a molecular ion peak at *m/z* 426 $[M]^+$. The molecular formula $C_{30}H_{50}O$ was deduced from its EIMS and NMR. The IR spectrum exhibited a hydroxyl (3,300 cm^{-1}) absorption band. The ^{1}H-NMR spectrum of compound **1** showed two olefinic methines [δ 4.69 (1H, s, H-29a) and 4.57 (1H, s, H-29b)], an oxygenated methine δ 3.20 (1H, dd, *J*=5.4, 9.9 Hz, H-3) and seven methyl groups [δ 1.68 (3H, s, H-30), 1.03 (3H, s, H-26), 0.96 (3H, s, H-23), 0.95 (3H, s, H-27), 0.83 (3H, s, H-25), 0.79 (3H, s, H-28) and 0.76 (3H, s, H-24)]. The ^{13}C-NMR and DEPT spectra showed thirty signals and exhibited two olefinic methines [δ 150.7 (C-20) and 109.3 (C-29)], an oxygenated methine [δ 78.8 (C-3)], and seven methyl groups [δ 28.0 (C-23), 19.3 (C-30), 18.0 (C-28), 16.1 (C-25), 16.0 (C-26), 15.4 (C-24) and 14.5 (C-27)]. As a result, compound **1** was identified as 3β-hydroxylup-20 (29)-ene (lupeol) by comparing its spectroscopic data with the previously reported data [12, 15].

Compound **2** was identified as stigmast-5-en-3-β-ol (β-sitosterol) through the comparison of spectroscopic data with the previously reported data [8, 11].

Compound **3** was obtained as white amorphous powder

and a molecular ion peak at *m/z* 456 $[M]^+$. The molecular formula $C_{30}H_{48}O_3$ was deduced from its EIMS and NMR. The IR spectrum exhibited a hydroxyl (3,420 cm^{-1}) and an olefine (1,690 cm^{-1}) absorption band. The ^{1}H-NMR spectrum of compound **3** showed an olefinic methine [δ 5.13 (1H, s, H-12)], an oxygenated methine [δ 3.02 (1H, dd, *J*=4.9, 10.0 Hz, H-3)] and seven methyl groups [δ 1.05 (3H, s, H-23), 0.92 (6H, s, H-26, 27), 0.87 (3H, s, H-24), 0.82 (3H, d, *J*=6.3 Hz, H-30), 0.76 (3H, s, H-29) and 0.68 (3H, s, H-25)]. The ^{13}C-NMR and DEPT spectra showed thirty signals and exhibited an olefinic methine [δ 124.9 (C-12)], an oxygenated methine [δ 77.1 (C-3)] and seven methyl groups [δ 28.6 (C-23), 23.6 (C-27), 21.4 (C-30), 17.3 (C-26), 17.2 (C-29), 16.4 (C-25) and 15.5 (C-24)]. These results, compound **3** were identified as 3-hydroxyurs-12-en-28-oic acid (ursolic acid) by comparing different physical and spectroscopic data with the previously reported data [18].

Compound **4** was obtained as amorphous yellow powder and a molecular ion peak at *m/z* 192 $[M]^+$. The molecular formula $C_{10}H_8O_4$ was deduced from its EIMS and NMR. The IR spectrum exhibited a hydroxyl (3,339 cm^{-1}), a carbonyl (1,704 cm^{-1}) and an aromatic C=C (1,566 cm^{-1}) absorption band. The ^{1}H-NMR spectrum revealed characteristic coumarin signals of two methines [δ 7.03 (1H, s, H-5) and 6.70(1H, s, H-8)], two aromatic methines [δ 7.82 (1H, d, *J*=9.3 Hz, H-4) and 6.14 (1H, d, *J*=9.3 Hz, H-3)], and a methoxy group [δ 3.88 (3H, s, OCH_3)]. The ^{13}C-NMR and DEPT spectra showed ten signals and exhibited a carbonyl group [δ 164.8 (C-2)], four methines [δ 146.6 (C-4), 111.6 (C-3), 109.9 (C-5) and 104.5 (C-8)], a methoxy group [δ 57.0 (C-6)], and four quaternary carbons [δ 155.9 (C-7), 152.4 (C-8a), 148.3 (C-6) and 112.0 (C-4a)]. Thus, based on all the above obtained spectral data with the previously reported data [16], the compound **4** was identified as 7-hydroxy-6-methoxycoumarin (scopoletin). This compound was isolated from *S. commixta* for the first time.

Inhibition of LDL oxidation

Inhibitory effects of isolates (**1-4**) on $CuSO_4$-induced oxidized low-density lipoprotein (ox-LDL) was measured by generation of TBARS. Among the isolates (**1-4**) tested, compound **4** showed significantly strong activity of ox-LDL inhibition with IC_{50} value of 10.2±0.1 μM and its activity was about 8-fold higher than a synthetic antioxidant, ascorbic acid (IC_{50}=84.1±0.3 μM) (Table 2). The other compounds (**1-3**) showed no inhibitory effects on LDL oxidation (IC_{50} >80 μM).

Table 2. Inhibitory activity of $CuSO_4$-induced LDL oxidation of compounds 1-4

Compounds	IC_{50} (μM)
1	>80
2	>80
3	>80
4	10.2±0.1
Ascorbic acid	84.1±0.3

It is reported that ox-LDL increases the adhesive properties of endothelium in a similar manner to effects of pro-inflammatory cytokines, resulting in endothelial dysfunction in the initial step of the atherosclerotic process [9, 25]. Because ox-LDL is a primary cause of endothelial inflammation, compound **4** might possess anti-inflammatory activity in vascular endothelial cells.

Effect of scopoletin on TNF-α-induced expression of adhesion molecules and adhesion of monocytes onto vascular endothelial cells

TNF-α is one of the major inflammatory cytokines that primarily targets vascular tissues [27]. Stimulation of endothelium with TNF-α causes up-regulation of endothelial ad-

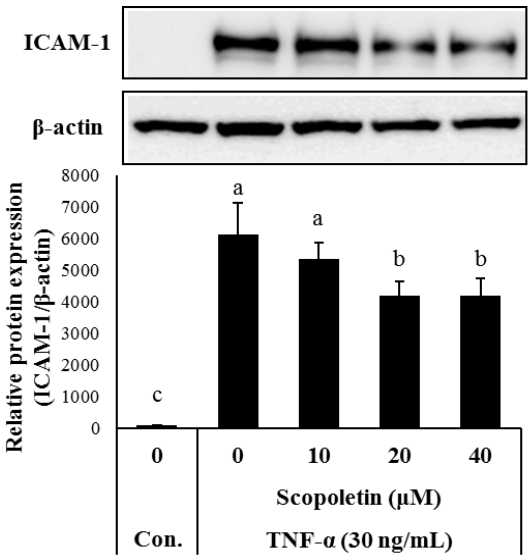

Fig. 2. Effects of scopoletin on TNF-α-induced ICAM-1 expression. EA.hy926 cells were pretreated with scopoletin for 2 hr and stimulated with TNF-α for an additional 18 hr. Whole cell extracts (20 μg) were subjected to Western blotting for ICAM-1. Bar graph (lower part) shows densitometric evaluation of ICAM-1 relative to β-actin. Results are expressed as mean ± SD (n=3). Different letters indicate a significant difference according to the ANOVA (p<0.05).

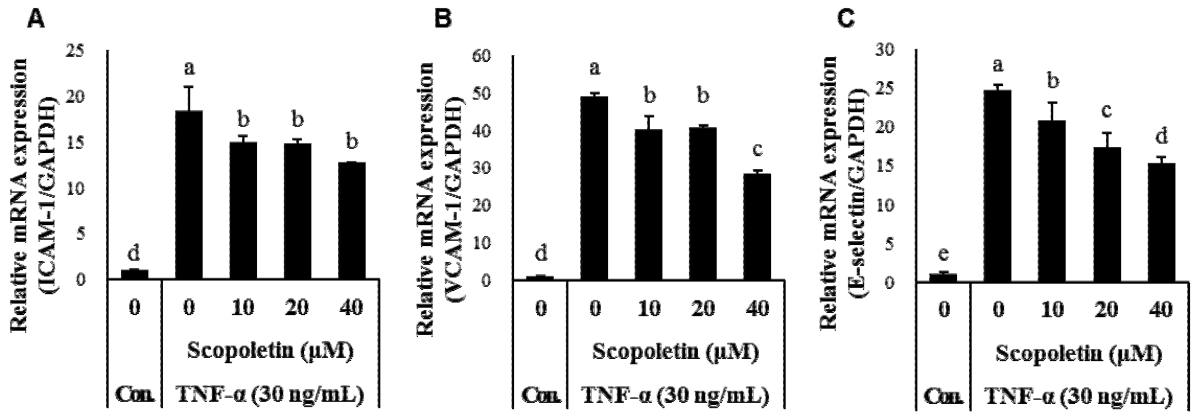

Fig. 3. Effects of scopoletin on TNF-α-induced mRNA expression of adhesion molecules such as ICAM-1 (A), VCAM-1 (B), and E-selectin (C). EA.hy926 cells were pretreated with scopoletin for 2 hr and then stimulated with TNF-α for an additional 4 hr. Total RNA was isolated from the cells and subjected to real-time PCR. Results are expressed as mean ± SD (n=4). Different letters indicate a significant difference according to the ANOVA (p<0.05).

hesion molecules such as ICAM-1, VCAM-1 and E-selectin, leading to adhesion of circulating monocytes with endothelial cells which have the potential to differentiate into tissue macrophages [3, 6]. We evaluated inhibitory activity of scopoletin against TNF-α-stimulated inflammation in endothelial cells. To conduct this study, EA.hy926 cells were treated for 2 hr with scopoletin prior to TNF-α stimulation for several hours (4-18 hr). TNF-α-induced ICAM-1 protein expression was markedly reduced by scopoletin treatment (Fig. 2). By 4 hr after TNF-α treatment, scopoletin inhibited

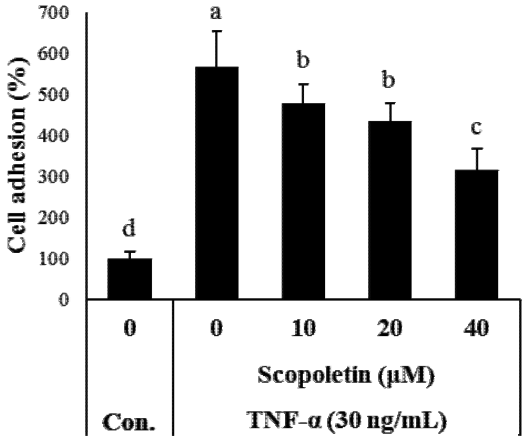

Fig. 4. Inhibition by scopoletin of THP-1 monocyte adhesion to TNF-α-stimulated endothelial cells. EA.hy926 cells were preincubated with scopoletin for 2 hr and then stimulated with TNF-α for an additional 18 hr. After staining of TNF-α-treated THP-1 cells with BCECF for 1 hr, the cells were added to EA.hy926 cells and incubated for 45 min. Results are expressed as mean ± SD (n=4). Different letters indicate a significant difference according to the ANOVA (p<0.05).

mRNA expression of VCAM-1 and E-selectin as well as ICAM-1 (Fig. 3). Also, scopoletin showed strong inhibitory effect on the adhesion of THP-1 monocytes to endothelial cells induced by TNF-α (Fig. 4). Adhesion level between THP-1 and EA.hy926 cells was determined by BCECF fluorescence. The TNF-α-treated THP-1 cells significantly increased BCECF fluorescence interacting with EA.hy926 cells, while the cells treated with scopoletin plus TNF-α decreased the TNF-α-induced cell adhesion in a dose-dependent manner. These data indicated that TNF-α significantly induced vascular endothelial inflammatory responses including expression of molecules related to cell adhesion, but this induction was suppressed by treatment with scopoletin.

In previous study, ginsenosides Rg2 and Rg3 as active components of *Panax ginseng* have shown to be involved in protecting against vascular inflammation through inhibition of cell adhesion and its molecules expression in inflammatory endothelial cells [6, 10]. Scopoletin also showed similar results as ginsenosides Rg2 and Rg3.

Effect of scopoletin on TNF-α-induced NF-κB signaling pathway in vascular endothelial cells

As next step, we further tested whether scopoletin regulated TNF-α-induced NF-κB signaling pathway in EA.hy926 cells. Scopoletin suppressed NF-κB translocation from cytosol to nucleus in TNF-α-stimulated endothelial cells (Fig. 5). It was also shown that IκBα phosphorylation was significantly reduced by scopoletin (Fig. 6). In particular, its expression level on treatment with scopoletin at 40 μM was similar to control cells. This results demonstrates that scopo-

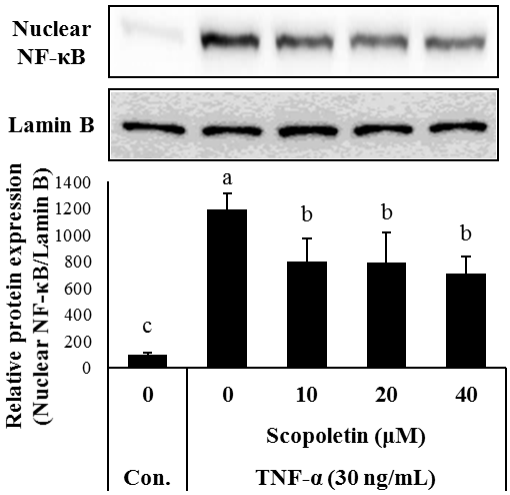

Fig. 5. Effects of scopoletin on TNF-α-induced NF-κB translocation. EA.hy926 cells were treated with scopoletin for 2 hr and then stimulated TNF-α for 30 min. Nuclear or cytosolic extracts (30 μg) were subjected to Western blotting for NF-κB translocation. Bar graph shows densitometric analysis of nuclear NF-κB relative to Lamin B. Results are expressed as mean ± SD (n=3). Different letters indicate a significant difference according to the ANOVA (p<0.05).

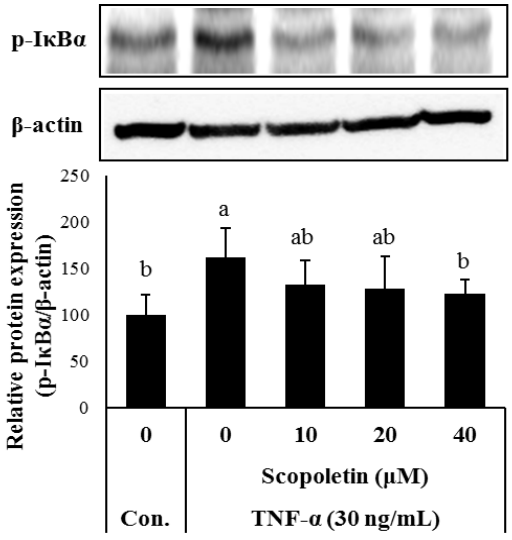

Fig. 6. Effects of scopoletin on TNF-α-induced IκBα phosphorylation. EA.hy926 cells were treated with scopoletin, followed by TNF-α treatment for an additional 30 min. It shows a representative Western blotting data for phospho-IκBα (p-IκBα). Results are expressed as mean ± SD (n=3). Different letters indicate a significant difference according to the ANOVA (p<0.05).

letin might block the nuclear translocation of NF-κB by suppressing the degradation of IκBα. It has been reported that NF-κB activation plays a crucial role in the vascular inflammation, resulting in the transcription of genes involved in endothelial inflammation [26].

In summary, four kinds of compounds were isolated from the $CHCl_3$-soluble fractions of *S. commixta*. The structures were identified as lupeol (**1**), β-sitosterol (**2**), ursolic acid (**3**), and scopoletin (**4**) by the physicochemical and spectroscopic data. In particular, scopoletin was isolated for the first time from this plant. The isolated compounds were evaluated for their LDL-antioxidant activities. Among them, scopoletin showed significant inhibitory activity against LDL oxidation. Also, in TNF-α-activated endothelial cells, scopoletin might cause inhibition of cell adhesion between THP-1 and endothelial cells by regulating NF-κB signaling pathway via inhibition of IκBα phosphorylation. As a result, scopoletin as an active compound of *S. commixta* may have anti-inflammatory activities on vascular endothelial cells and therefore further studies are needed to confirm *in vivo* bioactivity using animal models. Our study suggests that scopoletin have potential to be a new therapeutic candidate regulating the inflammatory vascular diseases such as atherosclerosis.

The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : 마가목 수피에서 분리한 scopoletin의 EA.hy926 혈관내피세포에서 NF-κB 신호전달을 통한 TNF-α로 유도된 혈관염증 저해 효과

강혜령[1] · 김효정[1] · 김보미[1] · 김선건[1] · 소재현[1] · 조수정[2] · 권현숙[1]*
([1]한국한의약진흥원, [2]경남과학기술대학교 제약공학과)

마가목은 한방에서 기침, 천식, 기관지 질환 등의 치료에 이용되고 있다. 본 연구에서는 마가목(*sorbus commixta*) 수피의 chloroform 분획물로부터 3종의 triterpenoid 화합물과 1종의 coumarin 화합물을 분리하였다. 분리된 화합물의 구조는 MS와 1D-, 2D-NMR분석에 의해 확인하였으며, 이들은 lupeol, β-sitosterol, ursolic acid와 scopoletin으로 구조동정되었다. 분리된 화합물 중 scopoletin은 마가목에서 처음으로 분리된 화합물이다. Scopoletin은 식물에 널리 분포하고 있는 물질로써 항염증 활성을 가진 기능성 화합물이다. 분리된 화합물들의 혈관 염증 억제에 대한 효과를 평가하기 위해 *in vitro*에서 LDL 산화 억제능을 평가한 결과, 분리된 화합물 중 scopoletin (IC_{50}=10.2 μM)이 강한 억제 활성을 나타내었다. TNF-α로 활성화된 인체혈관내피세포(EA.hy926)를 이용한 실험에서 scopoletin은 세포부착인자인 ICAM-1, VCAM-1, E-selectin의 발현을 저해하였고, THP-1 단핵구와 EA.hy926 혈관내피세포 간의 부착력도 약화시켰다. 뿐만 아니라, scopoletin은 TNF-α로 유도된 NF-κB 전사인자의 핵내 이동 및 IκBα의 인산화도 저해하였다. 따라서 마가목 추출물로부터 분리된 scopoletin은 NF-κB 신호전달의 억제를 통해 세포부착인자의 발현을 감소시키고, 단핵구의 혈관내피세포로의 부착을 억제시켜 혈관내 항염증 활성을 나타내었다. 이러한 실험결과, scopoletin은 혈관염증 반응으로부터 유도되는 죽상동맥경화증 치료를 위한 후보소재로서 이용될 가능성이 있다고 사료된다.